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Development of a bladder bioreactor for tissue-engineering in urology

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1 **Summary**

2 A urinary bladder bioreactor was constructed to replicate physiological bladder
3 dynamics. A cyclical low-delivery pressure regulator mimicked filling pressures of
4 the human bladder. Cell growth was evaluated by culturing human urothelial cells
5 (UCs) on porcine extracellular matrix scaffolds in the bioreactor and in static growth
6 conditions for 5 consecutive days. UC proliferation was compared with quantitative
7 viability indicators and by fluorescent markers for intracellular esterase activity and
8 plasma membrane integrity. Scaffold integrity was characterized with scanning
9 electron microscopy and 4,6-diamidino-2-phenylindole staining.

10

1.0 Introduction

Surgical repair for end stage bladder disease often utilises vascularised, autogenous, mucus-secreting gastrointestinal tissue to either replace the diseased organ or to augment inadequate bladder tissue. Augmentation cystoplasty is a urological procedure that involves the addition of viscoelastic ileal tissue to defective bladder tissue to improve functional bladder capacity. Postoperatively, the compliant smooth muscle of the bowel is often sufficient to restore the basic shape, structure and function of the urinary bladder, however lifelong postoperative complications are common. Morbidities that result from interposition of intestinal tissue may be divided into three broad areas: metabolic, neuromechanical, and technical-surgical. Metabolic complications are the result of altered solute reabsorption by the intestine of the urine that it contains. Neuromechanical aspects involve the configuration of the bowel; which affects storage volume and contraction of the intestine that may lead to difficulties in storage. Finally, technical-surgical complications involve aspects of the procedure that result in surgical morbidity.

ECMs are decellularised biological scaffolds commonly derived from porcine organs. After their preparation process decellularised ECM scaffolds may be seeded with autologous impermeable urothelial cells (UCs) *in vitro*. Cell-seeded scaffolds offer important advantages in urological settings when compared to gastrointestinal segments as UCs may provide a protective impermeable epithelial lining prior to *in vivo* implantation. Limitations associated with cell-seeded scaffolds are harvesting difficulties and low proliferative potential *in vitro*. Theoretically ‘bladder bioreactors’ may improve urothelial cell proliferation by preconditioning cell-seeded scaffolds in a simulated *in vivo* physiological environment. The objective of the present study was

- 1 to construct and evaluate a urinary bladder bioreactor for urological tissue-
- 2 engineering purposes.
- 3

1 **2.0 Materials**

2 **2.1 Construction of bioreactor**

3 Components were purchased from BOC gases® (United Kingdom and Ireland) unless
4 otherwise indicated. Primary constituents included a sealed pressure chamber with a
5 transparent window, pressurised gas containers and silicone tubing (Centre for
6 Applied Biomedical Engineering and Research [CABER], University of Limerick,
7 Ireland). The principal aim of the bioreactor was to increase hydrostatic pressure on
8 cell seeded ECM scaffolds from 0-10cm H₂O via a cyclical low-delivery pressure
9 regulator over preset durations. Urothelial cell viability and proliferative activity on
10 ECM scaffolds were measured and compared for 5 consecutive days in a control and
11 bioreactor experimental group by quantitative viability indicators.

12

13 **2.2 Culture and expansion of UCs**

14 Cell culture materials were obtained from Innovative Technologies in Biological
15 Systems, Bizkaia, Spain (Innoprot®) unless indicated.

16 1. UCs were initially cultured under standard cell culture conditions in a
17 humidified atmosphere of 95% air and 5% CO₂ at 37°C in T-75 vented cap
18 flasks (Sarstedt Ltd, Wexford, Ireland).

19 2. The cell line was grown to confluence in urothelial cell medium (UCM) and
20 supplemented with 1% urothelial cell growth supplement (UCGS) and 1%
21 penicillin/streptomycin solution (P/S solution). For expansion, cells were fed
22 every 48 hours until 85-90% confluency, which was usually achieved after 7-8
23 days.

24 3. Confluent cells were harvested by incubation with 0.0025% trypsin/ 0.5mM
25 EDTA Solution (T/E solution). Trypsin was neutralised with Trypsin

Neutralisation Solution (TNS) that contained buffered saline solution, 10% foetal bovine serum (FBS) and was calcium and magnesium free. The cells were then centrifuged for 5 minutes (1000rpm) and gathered. Passage 4 cells were used in the study.

2.3 Measurement of urothelial cell viability

Cell viability was assessed and quantified with the Alamar Blue® cell viability reagent (Invitrogen, Ireland) for 5 consecutive days. In the presence of viable cells the dye reduces, turns red and becomes highly fluorescent.

1. One tenth of the volume of alamarBlue® reagent was added directly into culture medium based on the protocol illustrated in Table 1.
2. Proliferation rates in both experimental groups were measured as a fold increase in the number of viable cells per day. To eliminate the potential for intervention bias between both groups, 2 control groups were created. Control group 1 consisted of increasing cell densities cultured in static growth conditions. Control group 2 consisted of increasing cell densities cultured in the bioreactor under static conditions (i.e. without the influence of cyclical pressure). Calibration curves with linear fit were applied to both control groups (Fig. 1)

2.4 Scanning electron microscopy (SEM)

To assess the structural integrity of UBM scaffolds in the bioreactor SEM was performed before and after 5 days on UBM with and without cells. The samples were sputter coated with a thin layer of gold and palladium.

2.5 4-6-Diamidino-2-phenylindole (DAPI) staining

DAPI nucleic acid staining (Invitrogen®, Dublin, Ireland) was performed to confirm the presence of cell nuclei in the bioreactor group after 5 days of growth (see note 1).

2.6 Measurement of fluorescence activity

‘Viability/cytotoxicity’ fluorescence assays were performed by means of three-dimensional confocal microscopy on both experimental groups to compare cell viability after 5 days of culture (LSM 700, Carl Zeiss Microimaging, Gottingen, Germany). Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein-AM to the intensely fluorescent calcein (see note 2).

EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells.

1 **3.0 Methods**

2 **3.1 Bioreactor and physiological intravesical pressures**

- 3 1. A manometer was placed in the sealed pressure chamber for the duration of
4 the study to accurately monitor pressures within the bioreactor throughout the
5 entire experimental time period.
- 6 2. Cyclical filling pressures of the human bladder were mimicked by connecting
7 a low delivery pressure regulator, with a pressure adjusting screw, to the gas
8 container (95% air and 5% CO₂) that increased the fluid pressure on the cell-
9 seeded UBM scaffold within the bioreactor from 0-10 cm H₂O (Fig. 2).
- 10 3. Application of cyclical pressure over a preset duration resulted in mechanical
11 strain on the cell-seeded ECM scaffold. Voiding pressures were mimicked by
12 releasing the infused gas from a 'Y-connector' silicon outlet source outlet
13 source over a period of approximately 10 seconds every 6-8 hours.

15 **3.2 Urothelial cell-seeding techniques in control and experimental group**

- 16 1. Specimens of urinary bladder matrix (UBM), a porcine extracellular matrix
17 graft, were cut into circles of 2cm diameter, transferred into 12-well tissue
18 culture plates and weighted with stainless steel rings also 2cm in diameter to
19 inhibit their lifting. Preparation of UBM has previously been described (see
20 note 3). Each stainless steel ring was autoclaved prior to insertion to ensure
21 sterility. UBM scaffolds were then seeded with urothelial cells ($2.5\text{-}5.0 \times 10^4$
22 cells/cm² per well).
- 23 2. Equally matched circles of UBM were inserted into a second modified 6-well
24 plate and transferred into the urinary bladder bioreactor for comparative

assessment. Luminal surfaces of UBM scaffolds were seeded with 5.0×10^4 cells/cm² per well and cultured in 2ml of UCM

3.3 Measurement of urothelial cell viability

1. After adding the alamarBlue® reagent, the solution was incubated for 4 hours at 37°C in a cell culture incubator that is protected from direct light (see note 4).
2. For greater sensitivity the extent of alamarBlue® dye reduction was quantified by fluorescence spectrophotometry (BioTek, Synergy HT Multi-Mode Microplate Reader). Proliferation rate was measured as a fold increase in the number of viable cells per day
3. Fluorescence was read using a fluorescence excitation wavelength of 540-570nm (peak excitation is 570nm). Fluorescence emission was read at 580-610nm (peak emission was 585nm). Fluorescence methods are more sensitive than absorbance methods and this method was applied to all experiments (see note 5).
4. Absorbance of alamarBlue® can also be monitored at 570nm, using 600nm as a reference wavelength value. Average 600nm absorbance values of the cell culture medium alone (background) were subtracted from the 570nm absorbance values of experimental wells. Background subtracted 570nm absorbance versus concentration of the test compound values were then plotted
5. Cells were plated in 100µL medium into 96-well tissue culture plates after performing cell number titration in the range of 40 to 10,000 for adherent cells and 2000 to 500,000 for suspension cells. A background control of 100µL of

medium without cells should be used. Subsequently 10µL of alamarBlue® was added into the medium and the cells incubated at 37°C overnight.

3.4 Scanning electron microscopy (SEM)

1. Initially, primary fixation was performed by immersing the sample in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 for 2 hours at room temperature or in a refrigerator at 4°C overnight.
2. Samples were washed for 5 minutes on 3 consecutive occasions in 0.1M cacodylate buffer (pH 7.4).
3. Secondary fixation was performed by immersing the sample in 1% osmium tetroxide (aqueous) pH 7.4 for 1 hour at room temperature in a lightproof container. The washing process was repeated in 0.1M cacodylate buffer 7.4.
4. Dehydration was achieved by the following method (see note 6):
 - 1 x 10min. in 25% ethanol,
 - 1 x 10min. in 50% ethanol,
 - 1 x 10min. in 70% ethanol,
 - 1 x 10min. in 85% ethanol,
 - 1 x 10min. in 95% ethanol,
 - 2 x 10min. in 100% ethanol,
 - 1 x 10min. 100% ethanol (EM grade).
5. Sputter coating was performed by applying a thin layer of gold and palladium over the sample with an automated sputter coater. This process can take up to 10 minutes.

3.5 4-6-Diamidino-2-phenylindole (DAPI) staining

1. To make a 5 mg/mL DAPI stock solution (14.3 mM for the dihydrochloride or 10.9 mM for the dilactate), dissolve the contents of one vial (10 mg) in 2 mL of deionized water (dH₂O) or dimethylformamide (DMF). The less water-soluble DAPI dihydro-chloride may take some time to completely dissolve in water and sonication may be necessary.
2. Counterstaining Protocol
 - Equilibrate the sample briefly with phosphate-buffered saline (PBS).
 - Dilute the DAPI stock solution to 300 nM in PBS. Add approximately 300 µL of this dilute DAPI staining solution to the coverslip preparation, making certain that the cells are completely covered.
 - Incubate for 1–5 minutes.
 - Rinse the sample several times in PBS. Drain excess buffer from the coverslip and mount.
 - View the sample using fluorescence microscopy with appropriate filters.

3.6 Measurement of fluorescence activity

1. Media was aspirated and the culture vessel washed with DPBS. Five millilitres of DPBS were added into a 15ml centrifuge tube followed by 10µl of ethidiumhomodimer-1 (Ethd-1) and 10µl of Calcein to the tube, (concentrations may change depending on the sample size).
2. The newly mixed solution was added to the cell sample and placed in a refrigerator at 4°C for 10minutes. After this timeframe, the solution was aspirated and the sample washed with DPBS. A small amount of DBPS was left on the sample to prevent dehydration.

4.0 Notes

- 1 DAPI staining was performed by dissolving 10mg of DAPI into 2mL of deionised water and protecting the solution from light. Subsequently, 300µL of the diluted DAPI staining solution was added to the cell-seeded scaffolds. The samples were then incubated for 5 minutes and evaluated with fluorescence confocal microscopy (LSM 700, Carl Zeiss Microimaging, Gottingen, Germany).
2. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm).
3. The harvested porcine bladder was rinsed in normal saline to remove the inner luminal urothelial cell lining. The outer abluminal muscular layers were removed by manual delamination. The specimen was bisected along 1 side to form a sheet. Subsequently, the tunica serosa, tunica muscularis externa, tunica submucosa, and the muscularis mucosa were manually removed while preserving an intact basement membrane and lamina propria. The decellularization process was completed by soaking the matrix in buffered saline (pH 7.4), placing it in peracetic acid/4% ethanol for 2 hours and rinsing it in sterile buffered saline. Finally, sterilisation was achieved by exposure to ethylene oxide.
4. Sensitivity of detection generally increases with longer duration times; therefore samples with fewer cells should use incubation times up to 24 hours.

1 5. Assay plates can be wrapped in foil, stored at 4°C and read within 1-3 days
2 without affecting the fluorescence or absorbance values.

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5 6. It takes approximately 40 minutes to achieve a critical dry point and the
6 sample can then be mounted onto a metal stub with a double sided carbon
7 tape.

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1 **Figure Legends:**

2 **Figure 1:**

3 A: Calibration curve for human urothelial cells cultured under static growth
4 conditions. B: Calibration curve for human urothelial cells cultured in the bioreactor
5 under static growth conditions.

6 Legend: Applying standard curves eliminated intervention bias between both
7 experimental groups.

8

9 **Figure 2:**

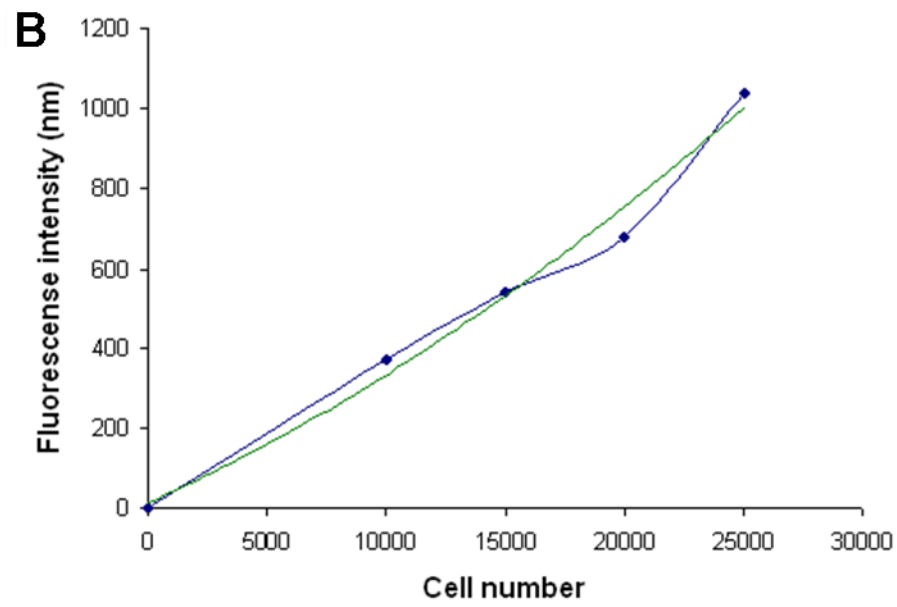
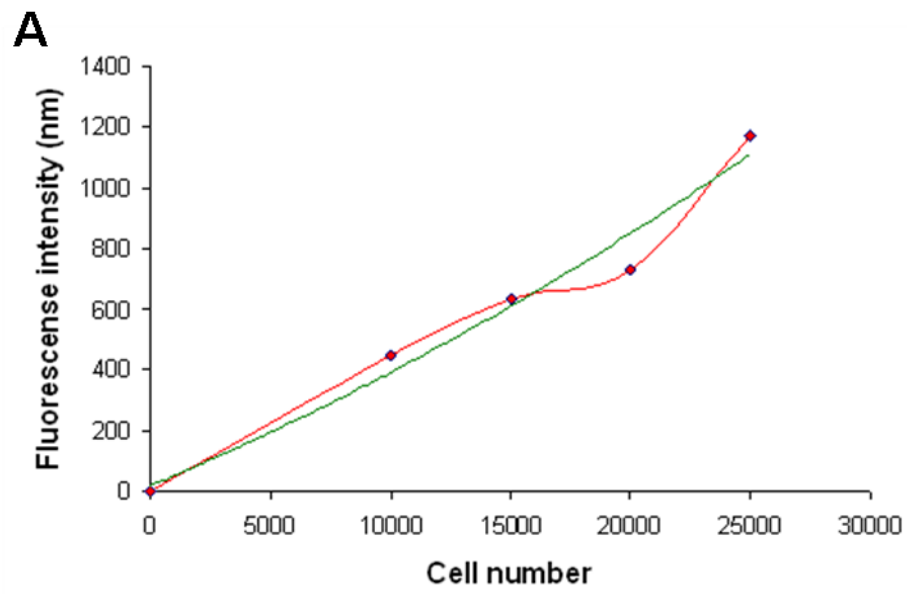
10 Photograph of bioreactor chamber and ECM scaffold exposed to cyclical
11 physiological urinary bladder pressures.

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Format	Volume of Cells & Medium	Volume of 10x alamarBlue® to add
Cuvette	1mL	100µL
96-well plate	100µL	10µL
284-well plate	40µL	4µL

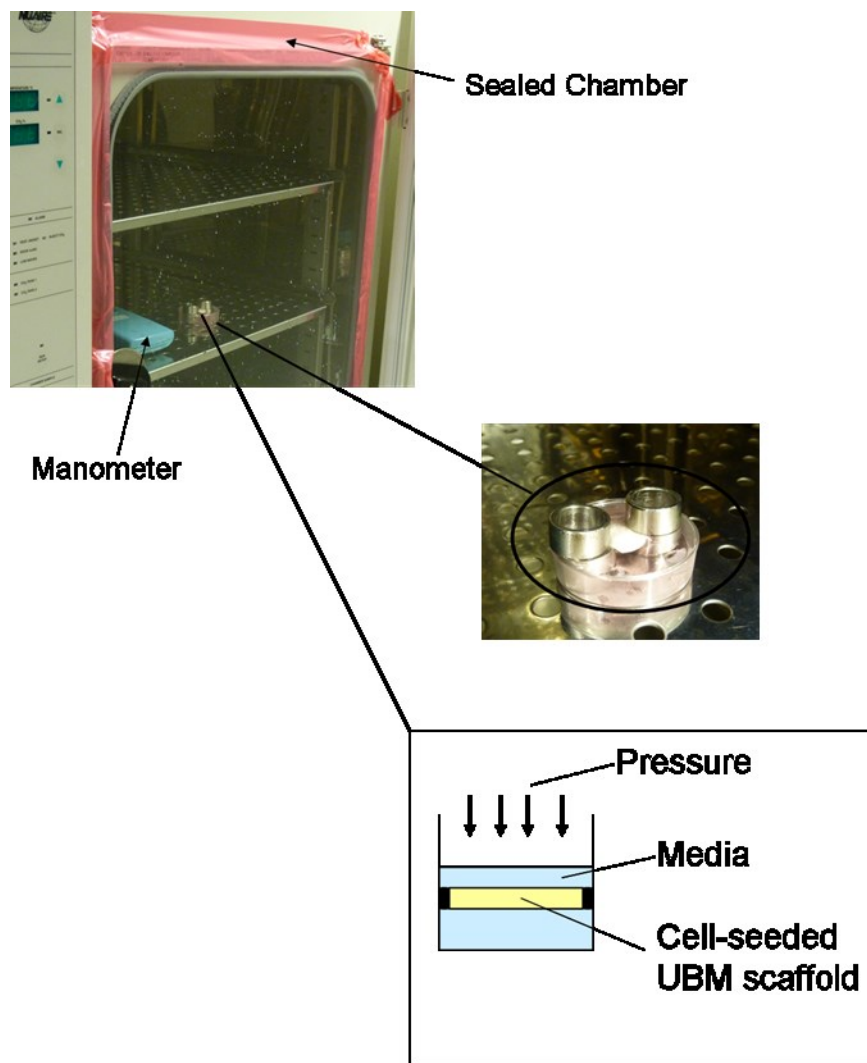
13

14 **Table 1:** AlamarBlue® protocol for assessing viable cell numbers



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2 Figure 1



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2 Figure 2